

# Tetrahydropyrimidine derivatives inhibit binding of a Tat-like, arginine-containing peptide, to HIV TAR RNA in vitro

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**Abstract** The ability of a small molecule, 2-methyl,4-carboxy,5-hydroxy-3,4,5,6-tetrahydropyrimidine (THP(A)), which accumulates intracellularly in various streptomycetes, to inhibit the interaction of Tat peptide (R52) with TAR RNA is presented. Using gel-shift assay, we found that the inhibition constant  $K_i$  of THP(A) is 50–100 nM, which is in the range of the binding constants of Tat peptide and protein. THP(A) is  $\sim 10^6$  times more tightly bound than the free L-arginine. The high binding affinity may be attributed to the special delocalized positive charge on the NCN group and the hydroxyl group at the 5 position of this molecule. A model for THP(A)-TAR interaction, analogous to the arginine guanidinium group-TAR interaction, is presented. The relatively high uptake of THP(A) by mammalian cells warrants in vivo Tat/TAR inhibition studies.

**Key words:** THP(A)/TAR-RNA binding; HIV-1-Tat/TAR inhibition; 2-Methyl,4-carboxy,5-hydroxy-3,4,5,6-tetrahydropyrimidine, THP(A); 2-Methyl,4-carboxy-3,4,5,6-tetrahydropyrimidine, THP(B); THP(A) uptake by mammalian cells

## 1. Introduction

Replication of human immunodeficiency virus (HIV) is critically dependent on two viral regulatory proteins, Tat and Rev. Tat is required in the early viral life cycle for efficient transcription of the viral genome. Tat acts by binding to an RNA stem-loop structure, the trans-acting responsive element (TAR), located at the 5' end of the viral mRNA (Fig. 1) [1–3]. A domain of basic amino acids mediate RNA recognition by Tat and peptides that correspond to this domain bind specifically to TAR [3–6].

The RNA-binding peptide from Tat has several unusual features in addition to its small size. Even though it binds specifically to TAR, the precise amino acid sequence required for specific RNA binding is flexible. To ascertain whether free amino acids can compete with the Tat peptide (R52: Tyr-Lys-Lys-Lys-Arg-Lys-Lys-Lys-Lys-Ala) for specific binding to TAR, inhibition of R52-TAR interaction by L-arginine, L-lysine and L-arginine derivatives was investigated by gel-shift assays [7]. The inhibition constant,  $K_i$ , for L-arginine was 4 mM, whereas the  $K_i$  for L-lysine was  $>50$  mM. This compares with the binding constants of Tat peptide and protein of 6–12 nM [7].

The conserved regions of biologically important RNAs often reside in loops, bulges and internal loops [8]. Structural studies

on RNAs have revealed that these regions are often stabilized by non-Watson-Crick base pairs. In HIV TAR, the three 'bulge' nucleotides are stacked between the upper and lower stem region, which forms base-pairs (Fig. 1). Upon binding of arginine or an arginine-containing peptide, a significant conformational change in the RNA is induced. The bound TAR RNA-arginine structure contains a new base-triple nucleotide in which the essential U in the bulge (U23) forms two hydrogen bonds with an essential Watson-Crick A27-U38 base pair in the major groove of the upper helix [9,10]. The nucleotides involved in the RNA base-triple interaction and in contacts to arginine are also those that are important for transcriptional activation in vivo [9].

The guanidinium group of the arginine side chain seems well suited for sequence-specific nucleic acid recognition. It is positively charged and can form base-specific hydrogen bonds in the DNA major grooves [11]. In RNA recognition, arginine can form a network of hydrogen bonds with the sugar-phosphate backbone [12] and can interact with the bases [13,14].

Because Tat and Rev are critical for viral replication, they are attractive targets for therapeutic intervention. Several previous studies have suggested that certain classes of small molecules, such as amino-glycoside antibiotics may interact with RNA in a sequence-specific fashion [15].

In our study of the control mechanism of actinomycin (Act D) biosynthesis by *Streptomyces parvulus*, we found and identified two tetrahydropyrimidine derivatives which accumulate intracellularly: 2-methyl,4-carboxy,5-hydroxy-3,4,5,6-tetrahydropyrimidine (THP(A)) and 2-methyl,4-carboxy-3,4,5,6-tetrahydro-pyrimidine (THP(B)) [16–18]. The simultaneous onset of their synthesis with that of Act D synthesis, leads us to believe that they may function in the self-defence mechanism of Act D-producing organisms. NMR and X-ray crystallography reveal that the THPs form zwitterionic molecules with the half-chair conformation (Fig. 2) [19]. The THPs are small, highly soluble organic molecules, neutral at physiological pH, which do not interfere with normal cellular functions. Thus, we anticipate that the amidine group of the zwitterion molecules, similarly to the arginine guanidinium group, can recognize a defined backbone conformation of the RNA by forming a specific network of hydrogen bonds with the sugar phosphate groups and/or interaction with the bases.

We present evidence that a small molecule, THP(A), can inhibit the interaction of Tat peptide (R52) with TAR. Using electrophoretic gel-shift assays, we found that the binding constant of R52-TAR was similar to the inhibition constant  $K_i$  for THP(A) of Tat-TAR binding. We predict a model for TAR-THP(A) or THP(B) interactions, analogous to the model proposed for the TAR-arginine guanidinium group interaction de-

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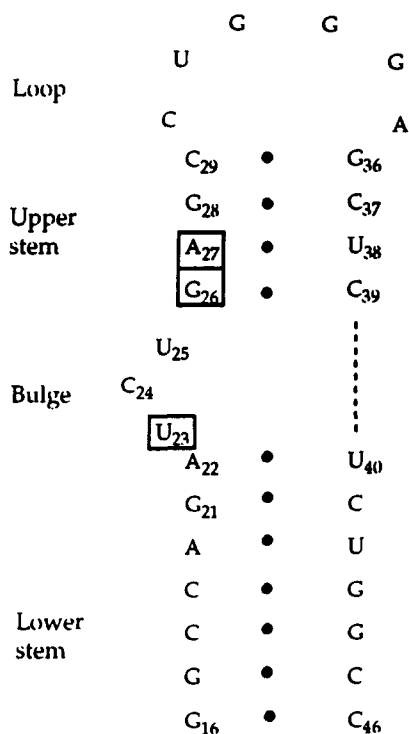


Fig. 1. Secondary structure of TAR RNA. Important sites for recognition by Tat are boxed. Data are from references cited in the text.

rived from NMR spectroscopy by Williamson and his colleagues [9,10].

## 2. Materials and methods

### 2.1. Tat peptide (R52) synthesis

A Tat peptide (R52) with a single arginine at position 52, which consists of nine basic amino acids and additional residues of Tyr at Lys NH<sub>2</sub> and Ala at Lys COOH termini, was used in this study - Tyr-Lys-Lys-Lys-Arg-Lys-Lys-Lys-Lys-Ala. The peptide was synthesized as previously described [6] by the Chemical Services Unit of the Weizmann Institute of Science. Tat (R52) was found to be pure by reverse phase high performance liquid chromatography (HPLC).

### 2.2. TAR-RNA synthesis and purification

TAR RNA was transcribed *in vitro* by T7 RNA polymerase [6] from synthetic oligonucleotide templates containing a 17 base double-stranded T7 promoter and a single-stranded TAR sequence coding for sense TAR RNA. The TAR RNA contained sequences +18 to +44 of the HIV LTR TAR site with additional GG at its 5' end, which increases the efficiency of transcription and CC at the 3' end to base pair with the Gs. The template containing the T7 promoter fused to the TAR coding sequences is as follows: 5'-CCG CAG AGA GCT CCC AGG CTC AGA TCT GGC CCT ATA GTG AGT CGT ATT-3' (the oligonucleotide was prepared by the Chemical Services Unit of the Weizmann Institute of Science). The RNA was purified on 10% polyacrylamide/8M urea gels, eluted from gels in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1% SDS, extracted twice with phenol, and precipitated with ethanol. Purified RNA was resuspended in sterile deionized water. The concentration of the radiolabeled RNA was determined from the specific activity of the [<sup>32</sup>P]CTP incorporated into the transcripts.

### 2.3. Gel-shift assay of RNA binding

Peptide and RNA were incubated together for 10 min in a 10  $\mu$ l binding reactions containing 10 mM Tris-HCl (pH 7.5), 70 mM NaCl, 0.2 mM EDTA and 5% glycerol, as previously described [7]. To determine the binding constants, 6 nM radiolabeled TAR-RNA was titrated

with at least five concentrations of Tat-peptide (R52): 60 nM of Tat was added to TAR to obtain 100% binding in the absence of inhibitors. Peptide-RNA complexes were resolved on 10% polyacrylamide [6]. Gels were electrophoresed at 200 V for 3 h at 4°C, dried and visualized by autoradiography. To determine  $K_i$  values, RNA-peptide bound and free RNA were quantitated by a  $\beta$  scanner.

(a) Inhibition of Tat (R52) binding to TAR RNA by THP(A) and THP(B) was measured by adding several concentrations of each compound to reaction mixtures containing peptide and RNA under conditions that gave approximately 100% binding in the absence of THP(A) or THP(B). The range of concentrations of THP(A) and THP(B) is indicated in Figs. 3 and 4.  $K_i$  is defined as the concentration of competitors required to decrease the fraction binding by 50%.

(b) Binding inhibition by L-arginine was measured as previously described [7] by adding several concentrations (in the range 3–120 mM) of L-arginine to reaction mixtures containing Tat peptide (R52) and RNA, as described above.

### 2.4. THP(A) and THP(B) biosynthesis

THP(A) and THP(B) were isolated and purified as previously described from *S. parvulus* [12,13]. THP(A) was recently obtained from *Streptomyces griseus* grown in a chemically defined medium supplemented with 0.5 M NaCl (63 mg/g dry wt) as the sole tetrahydropyrimidine derivative. It was isolated from other cell extract components and purified.

THP(A) and THP(B) were found to be pure by high field <sup>1</sup>H and <sup>13</sup>C NMR and by reverse phase HPLC.

### 2.5. Biosynthesis of [<sup>14</sup>C]THP(A)

We have previously found (Inbar and Lapidot, unpublished results) that [U-<sup>13</sup>C]aspartic acid can replace [U-<sup>13</sup>C]glutamic acid as the <sup>13</sup>C precursor for <sup>13</sup>C labeled THP(A) and THP(B) in *S. parvulus* cell culture. We have used this approach (with some modification) to prepare radioactive labeled [<sup>14</sup>C]THP(A) from [U-<sup>14</sup>C]aspartic acid in *S. griseus* cell culture.

### 2.6. Uptake of [<sup>14</sup>C]THP(A) by HeLa cells and human fibroblast cell line

HeLa cells (seeded at 15  $\times$  10<sup>6</sup> cell/ml) were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a 0.5 ml dish at 37°C. After 20 h, the medium was removed, cells were washed twice with 5 ml medium (without serum) and THP(A) was added to the cell culture at final concentrations of 0.05 to 1 mM. Cell growth was followed by counting, or alternatively, DNA synthesis was monitored by labeling cells with [<sup>3</sup>H]dThymidine (10  $\mu$ Ci/ml).

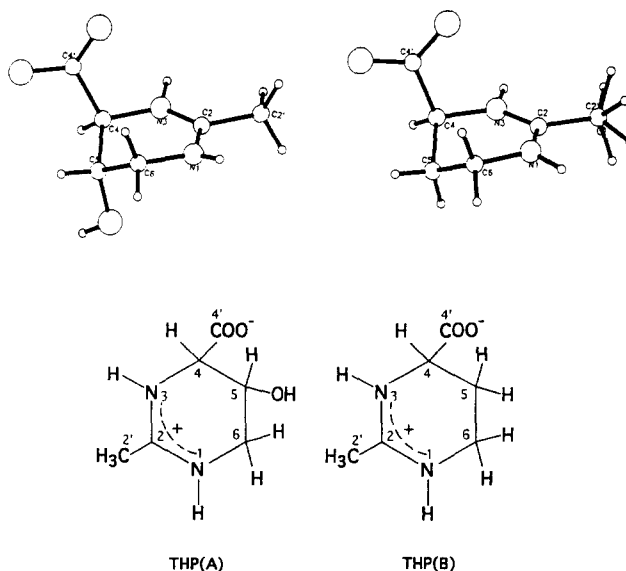


Fig. 2. Molecular structure of 2-methyl,4-carboxy,5-hydroxy-3,4,5,6-tetrahydropyrimidine (THP(A)) and 2-methyl,4-carboxy-3,4,5,6-tetrahydropyrimidine (THP(B)).

For the uptake experiments, [<sup>14</sup>C]THP(A) ( $5 \times 10^5$  cpm) was added to the washed cell culture at a final concentration of 0.5 mM and cells were cultured in DMEM (supplemented with 2% dialyzed FCS) for different incubation periods, as depicted in Fig. 4. The medium containing [<sup>14</sup>C]THP(A) was removed, cells were washed with phosphate buffered saline (PBS) and concentrated by centrifugation at 1500 rpm for 5 min at room temperature. Cells were lysed at 37°C for 18 h by a 100 μl NCS-tissue solubilizer (Amersham) and counted in lumax containing scintillation liquid.

Human fibroblast (R.G. foreskin cells) were grown similarly to HeLa cells. [<sup>14</sup>C] THP(A) was added to the culture at two final concentrations, 0.5 and 1 mM. The effect of uptake at these two concentrations were compared to that of Hela cells.

2.7. A suggested model for THP(A) and TAR interaction

The model of the THP(A)-TAR complex was constructed by manually docking the THP(A) molecule into the observed cavity in TAR, using interactive computer graphics (Biosym/ InsightII). Coordinates of the proposed TAR-arginine complex, based on NMR data, were generously given to us by Dr. James R. Williamson. The structure of THP(A) was taken from the crystal structure [19] except for minute changes in the torsion about the C-COO<sup>-</sup> and C-OH bonds. The model was not energy minimized.

3. Results

3.1. Inhibition of Tat peptide binding to TAR by THP(A)

The ability of THP(A), and to a much lesser extent of THP(B), to inhibit the interaction of Tat peptide to TAR-RNA was investigated. To simplify the analysis, we measured inhibition of Tat-peptide (R52) (a peptide containing a single arginine within a stack of lysines, see section 2) that binds to TAR. It was previously shown that this Tat peptide binds with the same affinity and specificity as the wild type Tat 49–57 peptide (Tyr-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ala) [7]. Using the

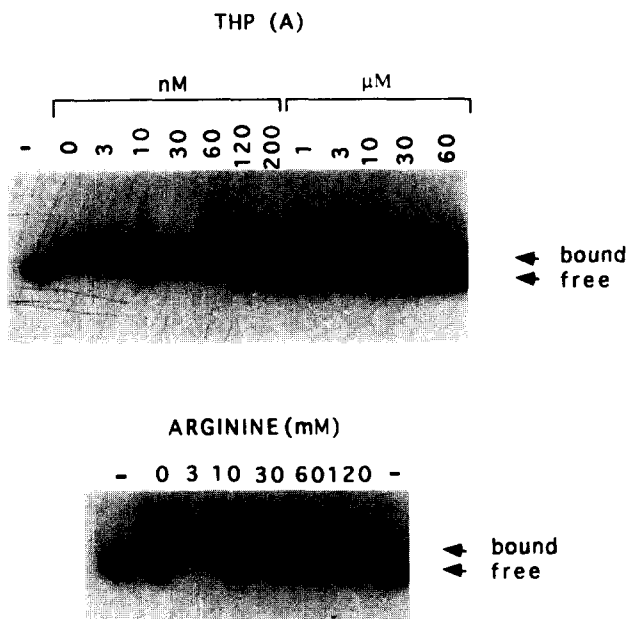


Fig. 3. Electrophoretic mobility-shift of Tat peptide (R52) binding to TAR RNA; effect of competitors. Specific inhibition of Tat peptide/TAR interaction by THP(A) or L-arginine are presented. Tat (R52) peptide (60 nM) was incubated with RNA (6 nM) in the presence of the indicated concentration of THP(A) (upper gel), or L-arginine (lower gel). Similar inhibition of Tat peptide/TAR interaction by THP(A) was obtained for at least three other experiments.

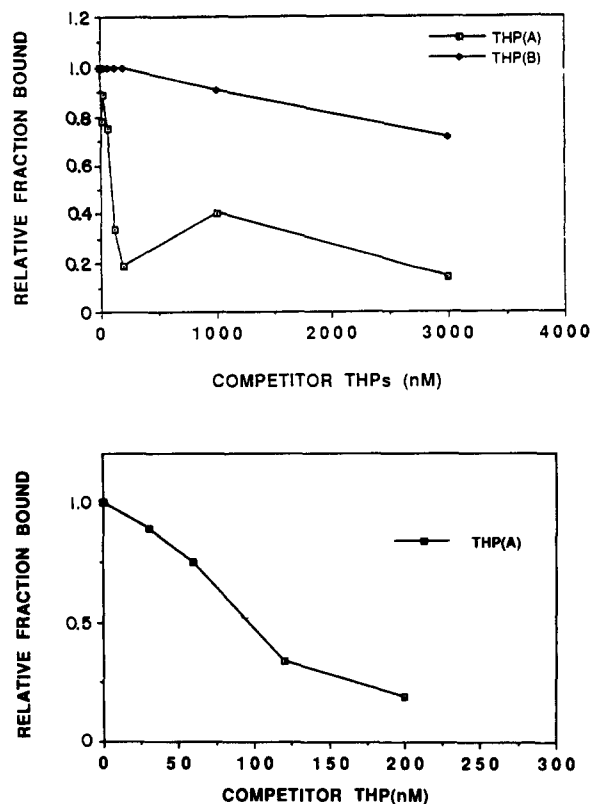


Fig. 4. (a) Binding curve with THP(A) was derived from the data in Fig. 3. Peptide-bound RNA and free RNA were quantitated from the gels presented in Fig. 3 with a β scanner, and the ratios of bound to free RNA, relative to absence of competitors, were calculated. The binding curve with THP(B) was similarly derived from inhibition of Tat/TAR interaction by THP(B) (data not shown), as described for THP(A) in Fig. 3. (b) Expanded view of Fig. 4(a) at THP(A) concentration of 0–200 nM.

gel-shift assay, we found that binding of Tat (R52) to TAR was strongly inhibited by THP(A) (Fig. 3). The inhibition constant  $K_i$  for THP(A) was in the range of 50–100 nM (Fig. 4). This compares with 100% binding of Tat (R52) to TAR at 60 nM Tat (R52), as measured in this study, and also with binding constants of 6–12 nM for Tat (R52) to Tat peptides, previously reported for 50% binding [7]. The high affinity of THP(A) to TAR-RNA is similar to that of the Tat peptide. Inhibition of Tat/TAR interaction by THP(B) was measured in the μM range. However, only partial inhibition was noted in this range (Fig. 4). The inhibition of Tat peptide (R52) binding to TAR by L-arginine was also measured in this study in comparison with THP(A) and THP(B). Inhibition by L-arginine in the mM range (Fig. 3) confirmed previous determinations [7]. The higher affinity of THP(A) compared to THP(B) may be due to the hydroxyl group at position 5, which can act either as a proton donor or acceptor and form hydrogen bonds with RNA bases. Moreover, the high affinity of THP(A) presented in this study, in comparison to that of free L-arginine and L-arginine analogues: argininamide and agmatine, which have a blocked or deleted carboxyl group [7] suggest that the carboxyl groups of THP(A) or THP(B) may take part in the interaction with TAR-RNA.

### 3.2. THP(A) is not toxic to mammalian cells – Uptake studies of THP(A) by mammalian cells

Two potential problems have concerned investigators who develop anticancer and antiviral drugs. The first is the ability of a charged molecule to diffuse across the uncharged membranes of the cells. The second, is the likelihood that compounds like oligonucleotides that managed to enter cells would be enzymatically degraded.

Since THPs are neutral at physiological pH, we anticipated that they could diffuse across the membrane, or even that their uptake by mammalian cells would be similar to that by *E. coli*, where high active transport occurs as a response to salt stress (Malin and Lapidot, unpublished results). We used radioactive labeled [ $^{14}\text{C}$ ]THP(A) for uptake studies by mammalian cells, e.g., HeLa, HL 60 and a human fibroblast cell line. We examined the cytotoxic effect of THP(A) on various mammalian cells, including HeLa cells and a human fibroblast cell line. Neither THP(A) nor THP(B) exerted a toxic effect on these mammalian cells in a culture medium at concentrations of up to 1 mM of each of the THPs.

For determination of uptake, samples of [ $^{14}\text{C}$ ]THP(A) (2,500 cpm/ $\mu\text{g}$ ) at a concentration of 0.5 mM were added for different incubation periods as depicted in Fig. 5. The uptake of THP(A) initially proceeded rapidly and was linear for about 6 hours before leveling off to a steady state. The intracellular level of THP(A) in HeLa cells at a steady state was  $1.0 \mu\text{mol}/10^6$  cells. Similar results were obtained for human fibroblast cells. However, when the extracellular concentration of [ $^{14}\text{C}$ ]THP(A) was 1 mM, its intracellular concentration was doubled after 6 to 18 hours of incubation with the labeled THP(A) (Fig. 5). During these long periods, cells were not degraded. These findings present a reasonably high uptake capacity of THP(A) by mammalian cells. Thus, *in vivo* inhibition studies of Tat/TAR interaction by THP(A) may be predicted.

### 3.3. A model for TAR-THP(A) interaction

A model for arginine guanidinium group interaction with TAR RNA was recently presented by Puglisi et al. [9]. The model, which is most consistent with NMR data, consists of a pair of hydrogen bonds between the guanidinium group and G26 in the major groove and hydrogen bonds to phosphates P22 and P23 that are favorably positioned in the bound structure.

The structural characteristics illustrated by the TAR bulge motif for TAR-arginine interaction [9,10] were used in this study to predict an analogous interaction between TAR-THP(A) and TAR-THP(B).

Upon binding of L-arginine to TAR-RNA, the conformational changes of RNA form a small cavity, which accommodates the guanidinium group of arginine [9]. The water-accessible surface of TAR shown in Fig. 6a reveals the binding cavity for the guanidinium group. THP(A) and THP(B) molecules

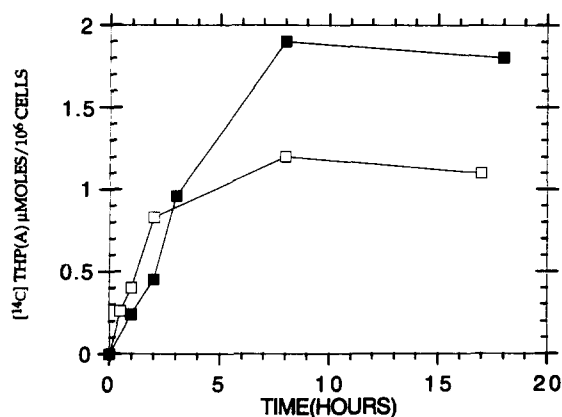


Fig. 5. Time dependence of [ $^{14}\text{C}$ ]THP(A) uptake by HeLa and human fibroblast cell lines. HeLa cells were incubated with 0.5 mM [ $^{14}\text{C}$ ]THP(A) and human fibroblast cell lines were incubated with 0.5 and 1 mM [ $^{14}\text{C}$ ]THP(A) for several time periods, as indicated.

were manually docked into this cavity. The NH hydrogens of the NH-C(CH<sub>3</sub>)-NH group in THP(A) (or THP(B)) are disposed similarly to the hydrogens of the NH<sub>2</sub>-C(NH<sub>2</sub>)-NH group of arginine (Fig. 6b). This structural similarity makes it possible to place the THP(A) (or THP(B)) amidine group inside the guanidinium binding cavity of TAR. THP(A) (or THP(B)) mimic only two of the four hydrogen bonds formed by the arginine guanidinium group, involving the phosphate of A22 and the O6 atom of G26. However, the THP(A) (or THP(B)) carboxylate group and, in particular, the hydroxyl group of THP(A) can also take part in the interaction with TAR (Fig. 6c). Most hydrogen bonds proposed for THP(A)-TAR interaction are in the range of 2.0–2.2 Å, as are those of the arginine-TAR complex. Our values are just an indication for possible hydrogen bonds, as the model was not energy minimized.

## 4. Discussion

### 4.1. The similarity of the arginine guanidinium group to the amidine group of the tetrahydropyrimidine derivatives, THP(A) and THP(B)

The similarity of the arginine guanidinium group to the amidine group of THP(A) or THP(B) (Fig. 2) prompted us to investigate the ability of THPs to interfere with the Tat/TAR interaction. Our previous studies indicated that both THP(A) and THP(B) are zwitterionic molecules with a delocalized  $\pi$  charge in the NCN group and form the half chair conformation [19] (Fig. 2) and both NMR and X-ray crystallography techniques indicated that the carboxyl group of THP(B) is in the axial position and that the carboxyl and hydroxyl groups of THP(A) are also in the axial position. The amidine group is positively charged, providing a favorable electrostatic environment for

Fig. 6. A model of interaction of THP(A) with the bulge region of TAR is presented. THP(A) is green, its amidine group nitrogens are blue, hydroxy and carboxylate oxygens are red, and RNA residues are yellow or brown. Hydrogen bond distances are given in Å. (a) The water accessible surface of TAR (yellow) reveals the binding cavity. The van der Waals surface (green) of THP(A) matches the size and shape of the cavity. (b) Comparison of the guanidinium group of arginine with the amidine group of THP molecules. The atoms of the similar moieties that interact in the binding cavity are shown as balls. (c) Interaction of THP(A) with TAR. The bond between the amidine group to G26 and P22, and the OH group to N7 and NH<sub>2</sub> group of A22, are shown, as well as the carboxylate group to A27. (d) Interaction of THP(A) with the triple mutant RNA (IV), C23-G27-C38. The hydrogen bonding between the amidine group and the hydroxyl group are as in the wild type (c), whereas the carboxylate group can participate in hydrogen bonding with the amino groups of C38 and C28 of the triple mutant (the model in (d) is presented at a different angle than that in (a)–(c)).



interaction with nucleic acids similarly to the guanidinium group of arginine. Thus, we surmise that the amidine group can donate hydrogen bonds to appropriately positioned acceptor groups, and the hydroxyl group of THP(A) and carboxylate group of THP(A) or THP(B) may also participate in the interaction with nucleic acids. Some evidence of the functional interaction of THP(A) with DNA stems from our recent finding that THP(A) protects a plasmid DNA from attack by restriction endonucleases (*EcoRI*, *AvaI* and *DruI*). THP(A) interacts with DNA and at  $10^{-4}$  M effectively protects the cleavage sites recognized by the restriction endonucleases (unpublished results). The crystal structure of *EcoRI*–DNA interaction [20] revealed that the bundle of four parallel helices of *EcoRI* is electrostatically attached to the phosphate group of the DNA backbone. Our results suggest that THP(A) binding to DNA prevents this interaction.

#### 4.2. A proposed mode for binding THP(A) to TAR.

In RNA recognition, the guanidinium group of the arginine side chain forms networks of hydrogen bonds with the sugar-phosphate backbone and interacts directly with the bases.

The results presented here suggest that both electrostatic attraction and hydrogen bonding are essential for THP(A) to interact with TAR, similarly to the binding of the guanidinium group of arginine to TAR. Moreover, the high affinity of THP(A) to TAR, which is similar to that of Tat (R52), but much higher than a single L-arginine, may be the result of other structural features involved in the THP(A)–TAR interaction. Based on NMR data of the conformational changes of TAR (31 nucleotides) in the presence of arginine, a model for arginine guanidinium group interaction with TAR RNA was presented [9,10]. It consists of a pair of hydrogen bonds between the guanidinium group and G26 in the major groove and hydrogen bonds to phosphates P22 and P23 that are favorably positioned in the bound structure. Their results [10] showed that the arginine interaction with TAR is stabilized by the formation of base triple U23–A27–U38. Mutants that disrupt the base triple destroy specific arginine binding to TAR, whereas the compensatory triple mutation, C23–G27–C38 that is isomorphic with the wild type, restores the binding. This suggests that arginine does not directly contact these bases.

In the model for the interaction of THP(A) and TAR, the binding to the atom of O6 G26 and to the phosphate group of A22 is similar to that suggested for the arginine guanidinium group (Fig. 6c). In addition, a three-centered hydrogen bond can be formed between the carboxylate group and the  $\text{NH}_2$  of A27, (Fig. 6c), which participates in the hydrogen bonds of the base triple (Fig. 1 in [10]). The higher affinity of THP(A) compared to THP(B) and to free arginine may be explained by the hydrogen bonding of the OH group with N7 and with the  $\text{NH}_2$  group of A22. This additional binding does not exist between the guanidinium group and TAR. But the most significant feature in the predicted model is that the small cavity formed by RNA folding in the presence of L-arginine can accommodate either the THP(A) or THP(B) amidine group. It is the similar arrangement of the hydrogen bond donors in guanidinium and amidine groups that makes it possible to superimpose them on one another (Fig. 6b), allowing similar interactions with TAR. Thus, one may expect that on binding of THP(A) to TAR, the latter will undergo similar conformational changes as those of arginine–TAR binding. It is also worth noting that the isomor-

phic base triple C23–G27–C38 in the triple mutant (IV) [10] can form hydrogen bonds to the THP carboxylate group via the C23 and C38 amino group protons (Fig. 6d). Thus, THP(A) can make a total of five or six hydrogen bonds with the wild-type TAR or its triple mutant (IV), respectively.

In conclusion, the structural characteristics illustrated by the bulge motif presented by Puglisi et al. [9,10], is applicable in this study for THP(A) (or THP(B)) and TAR interaction. However, definitive specification of the binding of THPs to TAR must await NMR analysis.

The high affinity of THP(A) to TAR–RNA and the fact that THPs are not toxic to human cells or animals (data not shown) and their resistance to enzymatic degradation makes THP(A) a good candidate for further studies towards a new pharmaceutical. Preliminary studies have shown that THP(A) interferes with HIV replication in cultured cells (A. Lapidot, A. Litovchick, A. Gazit and A. Yariv, manuscript in preparation).

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